

## Transglucosidation by Honey Invertase<sup>1</sup>

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### INTRODUCTION

In an investigation of the minor sugars of honey at this laboratory, examination by paper chromatography indicated that several oligosaccharides are present. These have not yet been identified. Since honey is the product of the reaction of honeybee (1) invertase with nectar sugars [sucrose, glucose, and fructose (2)], a comparison is being made of the sugars of honey with the oligosaccharides produced by the action of this invertase on sucrose. Considerable attention has recently been given to the production of oligosaccharide intermediates by transfructosidation during the action of yeast invertase on sucrose (3-6), but no reports of intermediate oligosaccharides formed by the honey invertase-sucrose reaction have been found in the literature.

Comprehensive studies (7-9) of the properties of honey invertase demonstrated definite differences between the action of this enzyme and that of yeast invertase. The data are consistent with the view that honey invertase is a glucosaccharase, although it was not so stated. Gorbach and Schneiter (10) later concluded that honeybee sucrase is a glucoinvertase; the identity of their enzyme preparation, an extract of honeybees, and that occurring in honey was not demonstrated.

In this paper the action of honey invertase on several sugars is reported. That the enzyme is a glucoinvertase is confirmed by chromatographic examination of its action on melezitose, raffinose, sucrose, and maltose, its inhibition by glucose, and the structure of the principal trisaccharide formed during sucrose inversion.

<sup>1</sup> Report of a study made under the provisions of the Research and Marketing Act of 1946.

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## EXPERIMENTAL METHODS AND RESULTS

### *Honey Invertase*

The enzyme concentrate was prepared by the procedure of Nelson and Cohn (7) from 484 g. of an unheated fall-flower honey of the 1948 crop that had been stored in a refrigerator for 33 months.

### *Activity*

Preparation A (1.00 ml.), sucrose (1.50 g.), and 0.5 ml. of 2 *M* acetate buffer were adjusted to 10 ml., giving a pH of 5.8. Determination of reducing sugar (Shaffer-Somogyi) showed that 58% inversion was reached in 125 min. at 26°C. The enzyme preparation alone had a reducing action equivalent to 0.35 mg. glucose/ml. This material showed an  $R_f$  of 0.00 on the papergram; spray reagents showed that it contained ketose. Further dialysis did not lower its reducing value. Under similar conditions, 2 ml. of preparation B inverted 63% of the sucrose in 4 hr.

### *Paper Chromatography*

Descending chromatograms were made on Whatman No. 1 paper with the solvent mixture *n*-butanol 3, pyridine 1, and water 1.5 by volume (11). Generally 20–45 hr. irrigation at room temperature was used. Sugars were detected with three chromogenic reagents:

(a) Benzidine citrate, modified from Horrocks (12) benzidine-acetic acid reagent.

(b) Naphthoresorcinol-phosphoric acid was used as a spray for detection of ketose-containing sugars (13).

(c) TTC, a triphenyltetrazolium chloride reagent (14) was used for detection of reducing sugars.

### *Action of Honey Invertase on Individual Sugars*

*Sucrose.* To a solution of 8.35 g. sucrose in water, 2 ml. of 2 *M* acetate buffer at pH 5.7 and 5.55 ml. of the enzyme preparation A were added. The solution was adjusted to 50 ml., and after 128 min. at 25°C., it was heated to boiling to inactivate the enzyme. After cooling, it was chromatographed on a 36 × 160 mm. column of Darco G-60<sup>3</sup> and Dicalite Speedex (1:1) by the method of Whistler and Durso (15) to separate it into mono-, di-, and trisaccharide fractions. After evaporation of solvent, the three filtrate fractions shown in Table I were obtained.

Samples of each fraction were chromatographed on paper; initial spots of 0.002 ml. of a 15% solution were used. Table II shows the re-

<sup>3</sup> Mention of trade names does not imply endorsement or recommendation by the Department of Agriculture over similar products not named.

sults, together with information obtained from the chromogenic reagents used.

Glucose, fructose, sucrose, and six other components were separated. Of these latter only two, those of  $R_s$ <sup>4</sup> 0.57 and 0.77, were present in appreciable amounts. Fraction 3 consisted primarily of the  $R_s$  0.57 compound, to which we have assigned the structure 4-( $\alpha$ -D-glucopy-

TABLE I  
*Composition of Hydrolyzate of 8.35 Grams Sucrose by Honey Invertase as Shown by Chromatography on Carbon*

Fraction	Eluting solvent	Weight of fraction g.
1. Monosaccharides.....	3.0 l. water	5.520
2. Disaccharides.....	3.95 l. 5% ethanol	2.074
3. Higher saccharides.....	1.0 l. 50% ethanol	0.944

TABLE II  
*Components of Sucrose Hydrolyzate by Honey Invertase*

Identity	Fraction (Table I)	$R_s$ <sup>a</sup>	Ketose-containing <sup>b</sup>	Reducing <sup>c</sup>	Relative amount
—	3	0.26	+	—	±
—	3	0.47	?	+	±
Maltosylfructoside (16)	3	0.57	+	—	++
—	2	0.59	—	+	±
Maltose	2	0.77	—	+	+
Sucrose	2	1.00	+	—	++++
—	2	1.04	?	+	+
Glucose	1	1.34	—	+	++++
Fructose	1	1.59	+	+	++++

<sup>a</sup> Ratio of distance moved by spot to distance moved by sucrose.

<sup>b</sup> Reaction with naphthoresorcinol spray reagent.

<sup>c</sup> Reaction with TTC spray reagent.

ranosyl)- $\alpha$ -D-glucopyranosyl- $\beta$ -D-fructofuranoside. Evidence for this structure is given elsewhere (16). The compound  $R_s$  0.77 was probably maltose, as shown by color reactions on paper and chromatography in comparison with known sugars. Glucose, fructose, and sucrose were also identified on the paper in this manner. A comparison of Table II with the results of White and Secor (6) and White (14) shows that at least five of these oligosaccharides differ from those formed by the action of yeast invertase on sucrose. The compound  $R_s$  1.04 is the only one that

<sup>4</sup> Ratio of the distance moved by spot to the distance moved by sucrose on the same paper.

cannot be shown to differ from the corresponding saccharide listed by White and Secor.

*Maltose.* To 1.500 g. of maltose and 0.5 ml. of 2 *M* acetate buffer at pH 5.8 was added 1.5 ml. of honey invertase A. It was adjusted to 10.00 ml. Samples were withdrawn at intervals for determination of reducing sugar (Shaffer-Somogyi). Simultaneously 0.002-ml. portions were transferred to filter paper, steamed, and chromatographed.

Table III shows the relative amounts of the various sugars in the solution during the course of the hydrolysis. At least three oligosaccharides of  $R_f$  less than maltose were present during the reaction. As is true of all cases reported here, none was present in the enzyme preparation or the original sugar used. The  $R_G$ <sup>5</sup> values for the compounds shown in Table

TABLE III  
*Composition of Honey Invertase Hydrolyzate of Maltose as Indicated by Paper Chromatography*

Time, min.....	0	3.7	12	34	129	305	1545
Extent <sup>a</sup> of hydrolysis, %.....	0	—	2.1	—	26	51	99
Oligosaccharide 3 .....	—	—	—	—	—	±	±
Oligosaccharide 2 .....	—	±	±	±	±	±	±
Oligosaccharide 1 .....	—	++	++	++	+±	+	—
Maltose.....	+++++	+++++	+++++	+++++	++++	++++	+±
Glucose.....	—	+	+	++	+++	++++	+++++

<sup>a</sup> Calculated from reducing sugar determinations.

III are oligosaccharide 3, 0.028; oligosaccharide 2, 0.135; oligosaccharide 1, 0.265; maltose, 0.49; and glucose, 1.00. These may represent a homologous series of  $\alpha$ -1,4-linked glucose polymers. A plot of  $\log R_G$  against degree of polymerization gives a straight line, with, however, the polymer of five glucose units missing. Barker and Bourne (17) have related this function [as  $\log R_f$  (spot) —  $\log R_f$  (glucose)] with chain length in a series of  $\alpha$ -1,4-linked glucose polymers formed by the action of an *Escherichia coli* preparation on maltose. Jeanes *et al.* (11) earlier suggested this relationship in the chromatography of a series of oligosaccharides produced by the action of malt  $\alpha$ -amylase on amylose. Table III shows that the trisaccharide component ( $R_G = 0.265$ ) was formed early in the reaction and disappeared before maltose did; the highest

<sup>5</sup>  $R_G$  is the ratio of distance moved by spot to the distance moved by glucose on the same paper.

member of the series ( $R_G = 0.028$ ) was not detected until late in the reaction.

These results may be considered with those of Wallenfels and Bernt (18), who recorded five oligosaccharides produced by the action of a mold enzyme on maltose. Since they do not record  $R_f$  values, the individual compounds cannot be compared. They quantitatively determined the amounts of the various intermediate sugars and reported isomaltose and maltotriose as components. Isomaltose was not present in the honey invertase-maltose reaction, judging from the absence of a spot on the papergram at about  $R_G$  0.37.<sup>6</sup>

*Raffinose.* A solution of 150 mg. raffinose, 2 ml. honey invertase *B*, and 0.5 ml. of 2 *M* acetate buffer of pH 5.8 was adjusted to 10 ml. and incubated at 32°C. under toluene. Reducing sugar content at 3 days was 0.55 mg./ml., calculated as glucose. Papergrams showed the presence of melibiose ( $R_G$  0.35), galactose ( $R_G$  0.86), glucose ( $R_G$  1.00), and fructose ( $R_G$  1.22).

*Melezitose.* A solution of 150 mg. melezitose, 2 ml. honey invertase *B*, and 0.5 ml. 2 *M* acetate buffer of pH 5.8 was adjusted to 10 ml. and held at 32°C. Reducing sugar content at 3 days was 1.58 mg./ml., calculated as glucose, or about three times the amount found for raffinose. The only hydrolytic products found by paper chromatography were glucose and fructose.

*Starch.* Degradation by the enzyme preparation of soluble starch to compounds giving no color with iodine showed that an amylase was present. The presence of amylases in honey is well known.

*Glucose.* Incubation of 15 mg. glucose with 0.1 ml. honey invertase under toluene for 5 days at room temperature produced materials giving faint spots  $R_G$  0.36 and 0.53. Neither was observed in control glucose spots. In  $R_G$ , the latter corresponds to maltose; the former, to isomaltose.

*Melibiose, Lactose, and Cellobiose.* Incubation of 15 mg. of each of these sugars with 0.1 ml. honey invertase at 37° for 3 hr. resulted in no hydrolytic action, as shown on the papergram; hence  $\alpha$ -galactosidase,  $\beta$ -galactosidase, and  $\beta$ -glucosidase were low or absent.

#### *Effect of Glucose and Fructose on Sucrose Hydrolysis by Honey Invertase*

*Glucose.* A mixture of 15 mg. sucrose and 15 mg. glucose was dissolved in 0.1 ml. honey invertase *A*. A control was prepared without glucose.

<sup>6</sup> This value was found for a sample of isomaltose kindly supplied by Dr. Allene Jeanes.

After 15 min., samples were heated and papergrams prepared from each. A definite inhibition of inversion was apparent in the sample containing the added glucose. It was shown both as greater intensity of the sucrose spot and less intensity of the fructose and oligosaccharide spots. There were no obvious differences in the number or intensities of the oligosaccharide spots relative to each other. Inhibition by glucose therefore is real, as judged by actual intensities of sucrose spots.

*Fructose.* A similar experiment in which fructose replaced the glucose showed no visual difference between sample and control in the intensities of sucrose and glucose spots. A slightly greater intensity was noted for the reducing disaccharide of  $R_s$  1.0 (Table II) in the presence of fructose.

The greater inhibiting action of glucose over fructose in this concentration range had been observed by Nelson and Scottery (8).

#### DISCUSSION

The relative inactivity of the honey enzyme preparation against raffinose, its moderate activity against melezitose, its stronger activity against maltose and sucrose, and its inhibition by glucose all indicate that an  $\alpha$ -glucosidase is a principal component of the enzyme system. The presence of  $\alpha$ -maltosyl- $\beta$ -D-fructoside (16) and probably maltose during sucrose inversion is also evidence that the sucrose-inverting action is due to a glucoinvertase. Thus, formation of  $\alpha$ -maltosyl- $\beta$ -D-fructoside by transfer of glucose from sucrose hydrolysis to an intact sucrose molecule is consistent with this view. The presence of maltose might be due to any of three possibilities: It may be formed by transfer of glucose from sucrose hydrolysis to another glucose molecule, it may be a hydrolytic product of the action of a trace of fructosidase on maltosyl fructoside (16), or it may be formed by a synthetic action of an enzyme component on glucose from sucrose hydrolysis.

Only a little fructosidase activity was present in the honey invertase preparation, as shown by the slight hydrolysis of raffinose to melibiose. Whether the invertase and maltase activities were due to the same enzyme cannot be stated, since no effort was made to fractionate the enzyme preparation. The amylase activity normal to honey was found in the preparation. No  $\beta$ -glucosidase,  $\alpha$ -galactosidase or  $\beta$ -galactosidase activity was noted.

In its transfer of glucose from sucrose to an acceptor without phosphorylation, the enzyme is somewhat similar to the amylosucrase of Hehre and Hamilton (19). The products of the reaction are different,

however; a 1,4-linked glucose polysaccharide is formed by amylosucrase, whereas a trisaccharide is the principal synthetic product of the action of the honey enzyme on sucrose.

Nelson and Cohn (7), studying the rate of sucrose inversion by honey invertase, found that it differs from the course of inversion of sucrose by yeast invertase. Using polarimetry to follow the reaction, they reported that after a marked initial increase in reaction rate to a maximum at 10–15% inversion, the velocity gradually fell during the rest of the reaction. They of course had no knowledge of the formation of the trisaccharide  $\alpha$ -maltosyl- $\beta$ -D-fructoside in the amount of about 10% of the total sugars. Since this compound has a specific rotation (16) nearly double that of sucrose, its presence would lead to erroneously high values for sucrose, which would affect velocity calculations.

#### SUMMARY

Evidence is reported for the formation of six oligosaccharides during the hydrolysis of sucrose by a honey invertase preparation, five of which differ from those formed in the hydrolysis of sucrose by yeast invertase.

The glucoinvertase nature of honey invertase is suggested by its hydrolytic activity against melezitose and its inhibition by glucose, which is not an apparent effect caused by accumulation of nonreducing oligosaccharide intermediates.

The structure of the principal oligosaccharide formed in the honey invertase-sucrose reaction is consistent with the view that glucose transfer is an integral part of this reaction and is further evidence of the glucoinvertase nature of honey invertase.

The action of honey invertase upon maltose produces in addition to glucose a series of glucose oligosaccharides, as shown by paper chromatography.

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